Structure, Origin and Function of Cytoplasmic Constituents in *Amoeba proteus* with Special Reference to Mitochondria and Golgi Substance

With 8 figures in the text

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William L. Doyle

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Berichterstatter: Dr. S. O. Mast
„ Dr. C. W. Metz

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I. Structure.

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Introduction.

Numerous observations have been made by various investigators on the structure of cytoplasmic constituents in *Amoeba proteus* and some suggestions have been made as to their origin and function, but there is still great diversity of opinion concerning their structure and the evidence presented in favor of the suggestions concerning their origin and function is very inadequate. This will be demonstrated presently.

Mast (1926, p. 419) maintains that *Amoeba proteus* consists of a central, granular, fluid portion, "plasmasol"; a granular, solid layer surrounding the fluid portion, "plasmagel"; a very thin well differentiated, elastic surface layer or membrane, "plasmalemma" and usually a thin hyaline fluid layer between the plasmalemma and the plasmagel. He says (p. 374): "The plasmasol consists of a fluid in which the following structures are suspended: numerous minute spherical granules (alpha granules); about an equal number of slightly larger irregular granules (beta granules); bipyramidal crystals and rectangular plate-like crystals varying greatly in number and size; refractive spherical bodies which also vary in number and size; various food vacuoles and usually the nucleus and the contractile vacuole."

We have, with various methods, thoroughly investigated all these structures except the nucleus. The results obtained are presented in this series of papers.

Materials and Methods.

Two species were used, *Amoeba proteus* obtained from a strain which has been maintained in the Zoological Laboratory of the Johns Hopkins University for over fifteen years, and *Amoeba dubia* obtained from J. A. Dawson, New York University. Both were cultured on inorganic salt solutions (Hahnert, 1932 and Pace, 1933) plus rice or wheat and food organisms. Cultures were prepared weekly and they were usually used when four weeks old.

The observations on the structure of *Amoeba* were made on living and on fixed specimens. Normal living specimens were put on a slide in culture fluid under a cover-glass supported by a vaseline ring. Then the cover-glass was gently pressed down until they were slightly flattened. Specimens in such preparations remain in active condition for one to three weeks. During this time they

decrease in size and become so translucent that the details of all their constituents can be clearly seen under immersion objectives.

The observations on normal specimens were supplemented by similar observations on specimens treated with the following vital dyes: janus green B (Grübler and General Dyestuff), bismarck brown, brilliant cresyl blue, chrysoidine, trypan blue, neutral red (Vital, Ehrlich and Coleman and Bell), rhodamine, and benzene-azo-alpha-naphthylamine.

In preparing fixed material which contains chemically diverse structures, it is highly desirable that the techniques used in different methods be as nearly uniform as possible thruout. Otherwise it is difficult to compare the results obtained by the different methods. The cytological effects of most of the modern fixatives have been admirably described in the works of Hirsch and Jacobs (1925), Bělař (1927), Zirkle (1928—1933), Hertwig (1930), Schiller (1930) and Bauer (1933). These descriptions indicate that for cytoplasmic structures, formalin is an ideal fixative if it can be used in such a way as to avoid shrinking and swelling of the cell as a whole. With this in view many different mixtures were tested. Of these the following was found to be the best: (Culture medium) 8.4 cc.; (Formol C. P. neutralized with $MgCO_3$) 0.6 cc.; (phosphate buffer M/5, p_H 6.2) 1.0 cc.

This mixture does not measurably change the volume of amoebae and it preserves all the constituents of the cytoplasm, except the nuclear granules and the water soluble salts, in a form scarcely distinguishable from the living condition; and it produces comparatively minor chemical changes in these constituents, so that when a second reagent (which may act either like a mordant or a solvent) is applied it acts nearly as it would if it were applied to living cytoplasm except that, owing to the hardening action of the formalin, it produces less distortion.

Fixation with this formalin mixture was therefore a preliminary step in nearly all the cytological techniques used. In this way a high degree of morphological uniformity was obtained which was invaluable in comparing results obtained by the different methods used.

Millon reagent was used in testing for protein; Benedict reagent, Nylander reagent, Best's carmine method and Lugol solution were used in testing for carbohydrates; sudan III and IV dissolved in various mixtures of alcohol acetone and water, Flemming fixative (strong) with and without acetic acid, 1 % and 2 % osmic acid, commercial nile blue sulphate and pure nile blue sulphate

oxazone, the Regaud technique, Ciaccio's methods, alcohol, acetone, acetic acid, sodium hydroxide. nitric acid, and turpentine were used in testing for fatty substances; and the following techniques: Altmann's fuchsin, Regaud, Bensley-Cowdry, copper-chrome-haematoxylin, Schridde, Mann-Kopsch, Kolachev, Champy-Kull, Benda, Da Fano, Nassanov and Defrise modification of the Regaud technique were used in testing for mitochondria and Golgi substances.

Numerous specimens were studied by each of the methods used. The results obtained by the different methods were carefully compared. Nearly all of the conclusions reached are based upon results obtained by several different methods. They will be presented under several headings.

Alpha Granules.

Vonwiller (1918) designated the alpha granules 'kleinste Körnchen'. Mast says they are approximately 0.25 micron in diameter. They are so near the limit of microscopical vision that we could see nothing concerning their structure in living specimens, and our efforts to stain them were unsuccessful. It was consequently impossible unequivocally to identify them in fixed preparations. We could therefore ascertain nothing concerning either their structure, origin or function.

Beta Granules.

Mast (1926) maintains that the beta granules are about one micron in diameter and that they are uniformly scattered thru the cytoplasm, except at the surface of the contractile vacuole where they tend to form a layer. They were designated: Elementarkörnern by Greeff (1891), plastiduli fucsinofili by Zoja (1891), Endoplasmakörner by Nirenstein (1905), sphaeroplastes by Fauré-Fremiet (1907), and Vonwiller (1915), excretion granules by Metcalf (1910), and mitochondria by Arndt (1924). They are probably homologous with structures in other organisms called cytomicrosomes and plastosomes by Duesberg (1912) and cytosomes or cytome by Dangeard (1931).

In view of the multiplicity of terms applied to these bodies it is evident that there exists considerable uncertainty as to their attributes.

The outline of the beta granules can be very distinctly seen in living specimens. By observing them from various points of view we found that those which are suspended in the cytoplasm

are nearly spherical or ellipsoidal, but that those which are on the surface of the contractile vacuole are flattened at the surface of contact with the vacuoles and with each other (Fig. 1 a, c). Individual granules were repeatedly selected and studied continuously for several minutes under the oil immersion objective and the following was observed: They slowly change slightly in form. Local protuberances resembling small pseudopodia appear at intervals as short as five minutes but there is no locomotion. The spherical ones sometimes become ellipsoidal or rod-like in form and vice versa. When they are rod-like they look somewhat like the bacteria found in the cytoplasm and in the food vacuoles (Fig. 1 c). In unfavorable culture media, intense light and ultra violet radiation they round up or break up into smaller spheres. This was repeatedly observed under the oil immersion objective.

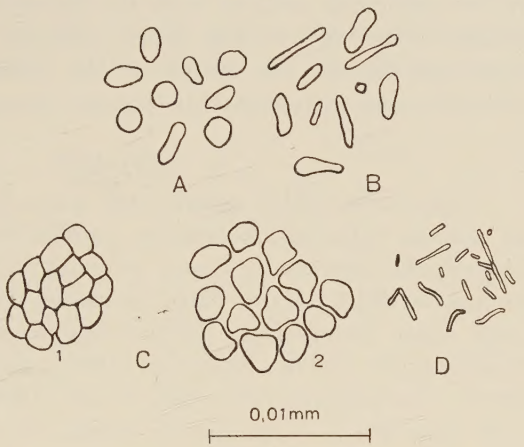

Fig. 1. Camera outline of beta granules (mitochondria) and bacteria in *Amoeba proteus*. A, beta granules found free in the normal living cytoplasm; B, beta granules after fixation with modified Regaud technique; C, beta granules on the surface of the contractile vacuole (1) in close contact with each other (2) with some space between; D, bacteria in the cytoplasm treated with Chrome-Osmic-Haematoxylin technique; mm, projected scale.

The facts that the beta granules become angular when they are in close contact and that they change in form indicate that they are plastic.

If amoebae are put into a 1 : 100.000 solution of janus green in culture fluid and then mounted with air bubbles as previously described, they live for several hours. If they are examined one-half to two hours after the addition of the stain all the beta granules and the bacteria, but no other structures, appear distinctly green. In the beta granules it can be clearly seen in optical section that only the surface is stained. This indicates that they have a differentiated surface layer similar to that in mitochondria.

In specimens treated with the mitochondrial methods given above numerous small bodies were found which have all the staining characteristics of mitochondria. These bodies are point for point like those described and called mitochondria by Fauré-Fremiet, Arndt, Zoja, and Vonwiller. They are, in size, number and distribution, like the beta granules seen in living specimens; but they are in general somewhat more elongated. This is less marked in the specimens treated with the Regaud technique than in those treated with any of the other methods. The elongated form is doubtless due to the action of the chemicals used. These facts therefore seem to demonstrate that the beta granules are mitochondria.

Crystals.

Schaeffer (1916) asserts that plate-like crystals are abundant in *Amoeba dubia* and absent in *Amoeba proteus* and that truncated bipyramidal crystals are very abundant in *Amoeba proteus* and rare in *Amoeba dubia*. Mast (1926) maintains that *Amoeba proteus* contains both types of crystals. Both forms were described in detail by Schewiakoff (1893), Schubotz (1905), Doflein (1907), Schaeffer (1916), Vonwiller (1918), and Ivanic (1924). Vonwiller maintains that they reach a maximum length of ten micra, that many of them are associated with large spheres "Kugeln" filled with fluid and that some of them have small blebs or Knöpfchen at some of their corners or edges. Edwards (1924) maintains that they are in vacuoles and Mast (1926) maintains that they are suspended in fluid in the vacuoles and that this fluid in these vacuoles becomes yellowish in solutions containing neutral red and consequently is alkaline.

Investigations concerning their chemical nature were made by Auerbach (1856), Carter (1856), Wallich (1863), Greeff (1891), Schewiakoff (1893), Stolc (1902), Schubotz (1905) and others.

There is no agreement among these investigators as to the chemical nature of the crystals. Carter (1856), and Schewiakoff (1893) concluded they are calcium oxalate; Wallich (1863) that they are carbonates or calcium salts; Entz (1879) and Rhumbler (1898) that they are excretory products; Stolc (1902) that they are leucine, Schubotz (1905) and Pollack (1928) that they are tribasic calcium phosphate, and it has been verbally suggested that they are cholesterol.

Not all authors distinguish between the two types of crystals found in the cytoplasm and it is not always clear what species

was used in the investigations. From the evidence to be presented it is evident that STOLC and SCHUBOTZ had reference to the plate-like crystals, whereas the other investigators probably dealt with the bipyramidal ones.

We made a thoro study of the crystals in a great many specimens of *Amoeba proteus* under oil immersion objectives, with and without polarized light. The results obtained lead to the following conclusions:

Practically all specimens of *Amoeba proteus* contain both plate-like and bipyramidal crystals. The number of both forms varies

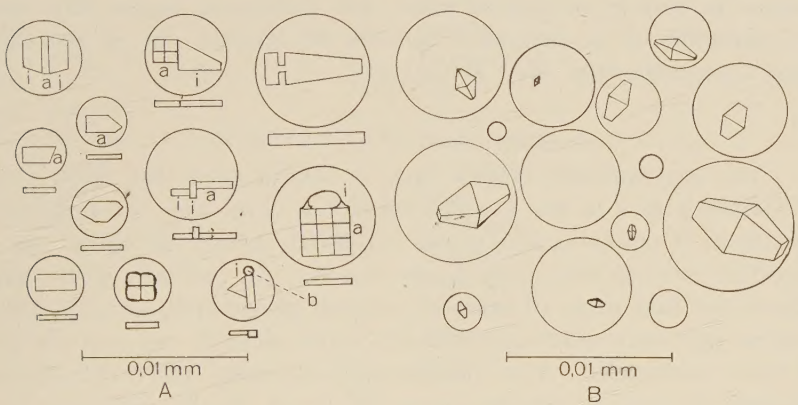

Fig. 2. Camera outlines of crystals in *Amoeba proteus*. A, plate-like crystals; circles, outlines of vacuoles; outlines within the circles, side view of crystals; outlines below circles, edge view of crystals; b, bleb; a, optically active substance; i, optically inactive substance; mm, projected scale. B, bipyramidal crystals, all optically inactive; circles, outlines of vacuoles. Note that the crystals vary greatly in size, that the relation in size between the vacuole and the crystals in it varies greatly and that some vacuoles have no crystals.

greatly in different individuals but the latter are always very much more abundant than the former. They are sometimes so abundant that the cytoplasm appears to consist largely of crystals. Both types are in vacuoles. The vacuoles contain hyaline substance in which the crystals are suspended, usually one in each vacuole. The vacuoles and the crystals vary greatly in size, and the size of the vacuoles in relation to the size of the crystals in them also varies greatly (Fig. 2 a, b).

The hyaline substance in which the crystals are suspended in the vacuoles becomes reddish yellow in neutral red. It is therefore alkaline. Its consistency is under some conditions so fluid that the

crystals move about freely and rapidly, i. e. Brownian movement is marked, and under other conditions so viscous that no Brownian movement whatever can be seen.

1. Plate-like Crystals.

The plate-like crystals are typically orthorhombic but they vary much in form and they frequently occur in hemimorphic combination with other types of crystals (Fig. 2a). They are all optically active but not on all axes and they exhibit parallel extinction of their optical activity. They resemble crystals of leucine in respect to crystal habit and extinction angles and they are insoluble in a saturated solution of leucine. It is therefore highly probable that they are leucine.

2. Bipyramidal Crystals.

The bipyramidal crystals vary in length from the lower limit of visibility to 7 μ, the average being 5 μ (Fig. 2b). No indication whatever of optical activity was observed in any of them. It is therefore obvious that they do not contain any optically active substance, and that since all oxalates, calcium phosphates and carbonates, leucine and cholesterol are optically active they do not contain any of these substances. The conclusions concerning their composition reached by Carter, Schewiakoff, Wallich, Stolc, Schubotz and Pollack are therefore not valid and they are not cholesterol.

To obtain further information concerning the chemical composition of the bipyramidal crystals we attempted to analyze them spectrographically and to ascertain their melting point and their solubility in various solutions.

a) *Solubility.*

Numerous observations were made under high power objectives on amoebae, some intact and some crushed, in various solutions. The results obtained are presented in table 1. By referring to this table it will be seen that the crystals are very soluble in all aqueous solutions, except concentrated ammonia and ammonium sulphate; that they are soluble in all mineral acids except concentrated sulphuric acid in which they decompose; that they are insoluble in concentrated organic acids, organic fat solvents and carbon disulphide; but that they are soluble in carbon disulphide saturated with ammonia. Their solubility is consequently like that of amino-acids. This therefore indicates that the crystals consist

Table 1.

Table showing solubility of the crystals of *Amoeba proteus* in various reagents. Dil, dilute; conc, concentrated; sat, saturated; aq, aqueous; alc, alcoholic; dec, decomposes; xxx, immediately soluble; ---, insoluble after several days; x, slowly soluble; -, soluble in one to two days. For solvents immiscible with water the specimens were dried by fixation in absolute alcohol.

Solution	Solubility	Solution	Solubility
H_2O	xxx	LiCl	xx
HCl (conc)	xx	$(NH_4)_2SO_4$	-
HCl (dil)	xxx	CH_3COOH (dil)	x
HNO_3 (conc dil)	xxx	CH_3COOH (conc)	---
H_2CrO_4 (dil)	x	HCOOH (conc)	---
H_3PO_4	x	$(COOH)_2$	-
H_2SO_4 (conc)	dec	Carnoy's	---
H_2SO_4 (dil)	xx	CS_2	---
NaOH (conc dil)	xxx	CS_2NH_3	xx
KOH (conc dil)	xxx	Alcohol (95%)	--
NH_4OH (conc)	-	Alcohol (70%)	x
NH_4OH (dil)	xxx	Ether	---
NaCl (sat)	x	Chloroform	---
$AgNO_3$	xx	Benzene	---
$Na_2S_2O_5$ (sat)	x	Toluene	---
KI (aq)	x	Glycerine	---
KI (alc)	--	Balsam	---
KNO_3	xxx	Xylol	---
K_2SO_4	xx		

of amino-acid. But if they do they must consist of glycine for, as demonstrated above, they are not optically active and glycine is the only amino-acid which is not optically active. The form of the crystals and the fact that solutions in which crystals have been dissolved give positive chemical tests for glycine also indicate that they are glycine. These tests were made as follows:

Approximately 5000 amoebae were put into 1 cc. of each of the solutions in which it was found that the crystals are readily soluble (Table 1) and left for several hours; the solutions were then filtered thru glass wool after which the filtrate of each was divided into two portions and sodium hypochlorite added to one and ferric chloride to the other. Positive tests for glycine were obtained.

If the crystals are glycine they must be insoluble in a saturated solution of glycine. It was found however that if amoebae are mounted in a saturated solution of glycine and torn open the crystals rapidly dissolve even when they are in close contact with crystals of glycine which, owing to evaporation, are increasing in size. It is consequently evident that the bipyramidal crystals are not glycine.

The facts presented clearly demonstrate, however, that they have a number of properties which are like those found in glycine. It is probable therefore that they consist of a compound of glycine.

b) *Melting Point.*

Ten to twenty amoebae were washed in distilled water and placed on a slide in a small drop of water. The slide was then put into a drying oven at 50° C and left eight to twelve hours. As a result the amoebae were dried and spread out in a very thin layer in which the crystals could be distinctly seen.

Minute crystals of a salt with a known melting point were now prepared by grinding some of the salt in an agate mortar and drying to constant weight at 110° C. A few of these minute crystals were then placed on the slide so that they and the crystals in the amoebae were visible in the same field under the low power of the microscope. The slide was now warmed from beneath by means of a micro burner attached to the stage of the microscope. This was continued until the crystals of the salt or those in the amoebae melted, sublimed or decomposed. The whole process was then repeated with other salts until one was obtained which melted at the same time that the crystals in the amoebae underwent a marked change.

It was found that when stannous chloride was used the crystals in the amoebae darkened a few seconds before the crystals of stannous chloride melted, that shortly after this they appeared to give off a small puff of smoke or vapor and that there remained a dark residue which became white when heated until the slide began to bend. It was concluded that the crystals in *Amoeba* decompose rather than melt and that this decomposition occurs at a temperature a little lower than the melting point of crystals of stannous chloride (246° C), i. e. that they decompose at about 240° C. Glycine crystals decompose at 275° C. The results obtained therefore support the conclusion presented above, namely, that the bipyramidal crystals in *Amoeba* are not glycine, but the facts that they decompose and leave a dark residue which becomes white with heat indicate that they consist of a combination of organic and inorganic substances, and the fact that the temperature required for decomposition does not greatly differ from that required for decomposition of crystals of glycine indicates that they probably consist of a salt of glycine.

c) *Spectrographic Analysis.*

To analyze a substance spectrographically it is highly desirable to have it pure. We therefore attempted to separate the bipyramidal crystals from the other substances in the amoebae. Two methods were used.

1. Large numbers of amoebae were killed in absolute alcohol and then put into each of various solutions in which the crystals are soluble and left 24 hours, after which the solutions were slowly evaporated. No crystals were obtained in any of them.

2. Numerous specimens were washed in distilled water then put into culture fluid which contained no calcium or magnesium, and left 48 hours with transfer to fresh culture fluid every 12 hours. Two hundred of these specimens were suspended in centrifuge tubes and rotated until they separated into two parts. The lighter parts were then collected and washed in two changes of distilled water, after which a drop of the last wash water was put into the depression of a clean graphite electrode in a spark apparatus aligned with a medium sized quartz spectograph. The circuit was now closed and the spectrum produced photographed. This was repeated with respectively the lighter and the heavier portions of the amoebae in the electrode which had contained the wash water.

Magnesium lines were found in the photograph of the spectrum produced by the heavier parts but in none of the others. In this spectrum the 279.5 mμ, the 280.2 mμ, the 285.2 mμ, the 292.8 mμ, and the 293.6 mμ, sensitive lines of the magnesium spark spectrum were fairly distinct: numerous lines representing other elements were found but the magnesium lines were the only ones not found in all of the spectra. During the process of centrifuging, nearly all the crystals and all the refractive bodies aggregated at the heavier end of the amoebae. The fact that the magnesium lines were found only in the spectrum of the heavier parts of the amoebae therefore indicates that either the crystals or the refractive bodies or both contain magnesium.

The experiment was consequently repeated with specimens containing many crystals but only very few refractive bodies. The results obtained were the same as those obtained in the preceding experiment. The magnesium is therefore probably in the crystals.

If the crystals contain magnesium and have properties similar to those of glycine, as demonstrated above, they probably are a magnesium compound of glycine. But if this is so, they are insoluble

in a saturated solution of this compound. We consequently made observations with methods described above, on the relative solubility of the crystals in saturated solutions of leucine, glycine, methyl glycine (sarcosine), trimethyl glycine (betaine) and methyl glycine plus magnesium hydrate respectively.

We found that they are soluble in all these solutions, but that the time required to dissolve in the solutions was much greater for methyl glycine plus magnesium hydrate than for any of the other substances tested. Consequently, while we have no conclusive proof as to what the crystals are, the facts that they contain magnesium, have several important properties of glycine, and are not readily soluble in a solution saturated with magnesium hydrate and methyl glycine indicate that they consist of a substance closely related to a magnesium salt of a substituted glycine.

Refractive Bodies.

Various investigators have observed relatively large, spherical, highly refractive bodies in the cytoplasm and in the food vacuoles in amoebae and they found that these bodies become deep red if the amoebae are stained with neutral red. Mast (1926) calls all these bodies refractive spherical bodies. Other investigators have used various other terms.

Those free in the cytoplasm were designated discoid ovules by Carter (1856), nucleated corpuscles by Wallich (1863), Glanzkörper by Greeff (1874), Glanzkügelchen by Stolc (1902), secondary nuclei by Calkins (1905), Eiweißkügeln by Vonwiller (1918), parasites by Mattes (1924), cysts by Taylor (1924), Golgi bodies by Brown (1930), nutritive spheres by Taylor (1932), and globules oleagineuse, spherules chromatiques, liposomes, and ergastome by Dangeard (1931, 1932, 1933). Bodies similar to those in the food vacuoles were designated Ballen and Körnerkonglomerate by Nirenstein (1905), Reservestoffe by Heironymus (Lafar, 1907), mitochondria by Horning (1925—1928), chromies and spherules metachromatiques by Dangeard (1931, 1932, 1933), vacuome by Volkonsky (1933), and spherical bodies by Mast and Hahnert (1935).

The extraordinary variety of names for the bodies clearly shows that the authors held widely divergent views as to their origin, structure and function. Thus Wallich, Calkins and Taylor (1924) considered them reproductive bodies; Carter, Greeff, Stolc, Vonwiller, Taylor (1932) and Dangeard, deutoplasmic bodies; and

Brown, Golgi bodies. Greeff, Stolc, Taylor (1932) and others held that they are homogeneous in structure and Carter, Wallich, Calkins, Vonwiller, Taylor (1924) and Brown held that they are not.

Despite evidence of occasional critical examination of the literature few authors have mentioned the views of previous investigators. This was probably due chiefly to the fact that different investigators used such widely different techniques that they did not recognize that the different terms used referred to the same structures.

We found that the refractive bodies in the cytoplasm differ radically from those in the food vacuoles. The former stain less quickly in solutions of neutral red, they are more complicated in structure and they are larger than the latter (the diameter of the largest being fifteen micra for the former and only ten for the latter). We will refer to those in the cytoplasm as refractive bodies and to those in the food vacuoles as vacuole refractive bodies.

1. Refractive Bodies.

Amoebae were put into a dilute solution of neutral red (approximately 1 : 50,000) and left until the refractive bodies were stained deep crimson. A specimen was then mounted in culture fluid under a cover-glass supported by a vaseline ring and greatly flattened by applying pressure to the cover-glass. The specimen was then studied under low and high power. It was seen that the central portion of the refractive bodies was definitely lighter than the outer portion and that the lighter portion was eccentric in some of the bodies. While being observed under moderate magnification (8 mm objective) pressure was applied to the cover-glass with a needle directly over the specimen. The refractive bodies spread out and as they spread the central portion became lighter until it was precisely differentiated from the outer portion which now consisted of a thick dark crimson layer. The pressure was now decreased, the diameter of the refractive bodies immediately decreased, and the crimson layer became thinner. This shows that the refractive bodies are highly elastic. The pressure was then gradually increased. The crimson layer became thicker and the central portion spread out until it had increased by about one-fifth in diameter, then it suddenly cracked into several pieces and a clear fluid flowed out of it (Fig. 3c). This shows that the central portion consists of a spherical fragile shell which surrounds a fluid substance.

In other preparations, the cover-glass was pressed down and released several times in rapid succession with the needle over a

region containing several refractive bodies in close contact with each other. The crimson layers of these refractive bodies fused and formed a continuous matrix thru which the central colorless masses were scattered (Fig. 3 b). It is therefore evident that the outer layer is definitely fluid but rather oily and viscous and that this is the only portion of the refractive body that stains with neutral red.

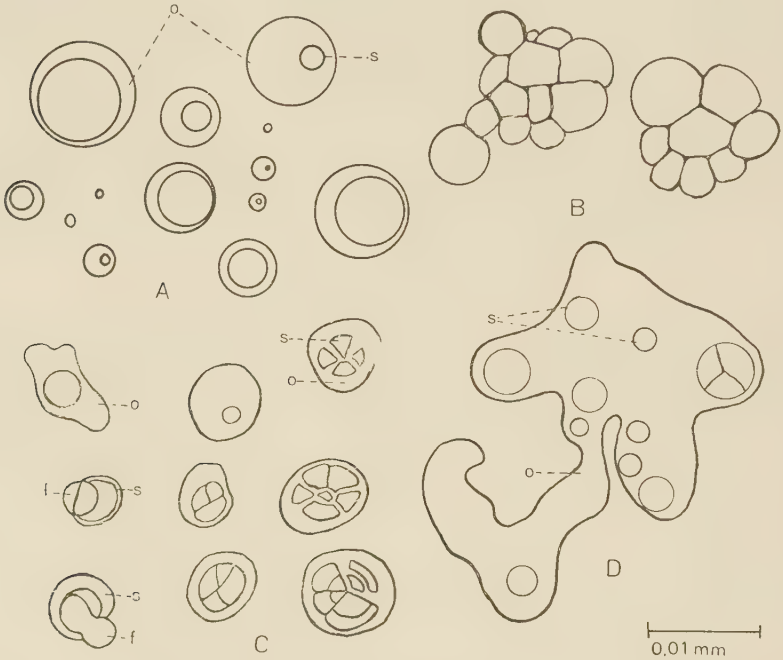

Fig. 3. Camera drawings of refractive bodies in the cytoplasm of *Amoeba proteus*. A, specimens free in the cytoplasm; o, outer layer (this stains crimson with neutral red); s, outline of fragile shell (this contains a fluid substance). Note that the refractive bodies vary greatly in size and that the outer layers vary greatly in thickness. B, specimens in contact with each other. Note that the outlines are angular indicating plasticity. C, specimens which had been subjected to pressure; o, outer layer; s, shell; f, fluid. Note that the shells have broken in some and cracked in others and that the central fluid was partly forced out of some which were cracked. D, outlines showing the effect of successive increase and decrease in pressure on a group of specimens in contact with each other. O, fused outer layers; s, shells; mm, projected scale. Note that one of the shells is cracked.

The refractive bodies consequently consist of an outer relatively fluid layer which stains crimson in neutral red and a central globular shell filled with a fluid which does not stain with neutral red.

Figure 3 shows that the size of the refractive bodies varies greatly and that the size of the shell in relation to the thickness of the outer layer also varies greatly.

Vonwiller (1918) maintains that the refractive bodies sometimes contain three or four differentiated masses. We observed this only in specimens which had been subjected to pressure. It is therefore probable that it occurs only in specimens in which the outer layers of refractive bodies have fused.

Taylor (1924) sectioned and stained what she called encysted amoebae and she maintains that she found in them cysts composed of a thin outer layer ("cyst wall") surrounding a droplet of "hatching ferment" in which are embedded a varying number of "chromosomes" and that these are "young amoebae". It is quite apparent from her description and more apparent after examination of her plates that the structures she observed were refractive bodies with cracked shells (probably broken by sectioning), that what she called chromosomes were fragments of the shell, that what she called hatching ferment was the fluid contained in the shell and that what she called cyst wall was the outer oily layer.

The question now arises as to what the composition of the different parts in the refractive bodies is. To answer this question numerous amoebae were treated with the reagents listed below and the effects on the various parts of the refractive bodies observed.

a) *Outer Layer.*

Specimens were treated with various fat stains. The outer layer was not stained by sudan dyes unless the amoebae were treated with phenol in accord with the method of Ciaccio (1925) before they were put into the dyes, then it became red. This layer became intensely blue in nile blue: and it became black in osmic acid, after which it did not bleach in hydrogen peroxide or turpentine. The fact that it does not stain with sudan dyes shows that it is not fat; but the fact that it stains in these dyes subsequent to treatment with phenol, and that it blackens with osmic acid and is not readily bleached, indicates that it is lipoidal: and the fact that it stains blue in nile blue shows that it contains fatty acid.

Amoebae were immersed in alcohol and thirty-five percent acetic acid respectively and left in each for twelve hours and then examined. The refractive bodies did not dissolve nor did they noticeably decrease in size. They were however less refringent after the treatment and they no longer stained with nile blue, osmic acid or

sudan dyes used in accord with Ciaccio's method. This indicates that the outer layer consists of a substance, which is soluble in fat solvents, dispersed in a substance which is not soluble in fat solvents, i. e. a lipid dispersed in a substance which is not lipid.

Numerous amoebae were either fixed in formalin and then put into methylene blue in sulphuric acid or put directly into methylene blue in sulphuric acid. In the latter, the refractive bodies aggregated and formed several different masses and the outer layers of the bodies fused and became blue-black. In those previously fixed in formalin they did not fuse but the outer layer became blue-black. This staining shows that the outer layer of the refractive bodies contains metachromatin, i. e. a substance composed of nucleic acid and an unknown base. Nucleic acids are frequently associated with proteins, the outer layer therefore probably contains protein.

Some specimens were put into acetone and then treated with Millon's reagent and others were put into acetic acid of various concentrations. In the Millon's reagent the outer layer of the refractive bodies gave a faint positive protein reaction. This shows that it contains protein. In acetic acid in concentrations up to thirty percent the outer layer of the refractive bodies dissolved. In higher concentrations it did not. This is precisely what would be expected if this layer consists of lipid substance and protein, for if it does the lipid would dissolve in the acetic acid in all concentrations used but the proteins would coagulate in the higher concentrations and disintegrate in the lower, and this would cause the outer layer to disappear in the weaker solutions but not in the stronger which is in accord with what was observed.

All of the results presented consequently indicate that the outer layer of the refractive bodies consists of a protein stroma which is impregnated with a lipid substance.

The fact that the outer layer contains protein lends some support to Vonwiller's contention that the refractive bodies are „Eiweißkügeln".

b) *Shell.*

The shell in the refractive bodies was tested for proteins, lipids and carbohydrates and it was studied in polarized light. No proteins, lipids or starches were found in it, but it was found that it is faintly optically active, that it reduces Nylander's reagent and that it dissolves in concentrated sodium hydrate. These reactions indicate that altho the shell is not starch, it is a carbohydrate.

c) *Fluid.*

Nothing was ascertained concerning the chemical composition of the fluid within the shell.

d) *Discussion.*

Brown (1930) as previously stated, concluded that the refractive bodies are Golgi bodies.

There is much controversy concerning the origin, structure, function and composition of the Golgi bodies. They are consequently not well defined. A considerable number of methods have been devised to test for them. Among the more important are those of Nassonov, Bensley-Cowdry, Champy-Kull, da Fano, Kolachev, Mann-Kopsch and Holmgren. All of these tests are based upon the chemical properties of substances in the Golgi bodies. The results obtained by these methods indicate that the Golgi bodies contain two substances. One which becomes black in osmic acid and does not readily bleach in turpentine, hydrogen peroxide or chlorine water but dissolves in fat solvents, and one which does not dissolve in fat solvents, e. g. trichloracetic acid. The former substance is often designated "O" and the latter "T". Beams (1931) and Tanaka (1932) have demonstrated that the "O" substance becomes red in solutions of neutral red and blue in nile blue and that the "T" substance does not stain in these solutions.

von Berger (1904) contends that the "O" substance is myelinoid or lecithin-like, Sjovall (1905) designates it myelinogen; Weigl (1911, 1912) says that is is lecithin: Rauber and Kopsch (1926) conclude that it is an albuminose or a peptone; and Tennent, Gardiner and Smith (1931) demonstrated in experiments on eggs of echinometra that it is a lipid characterized by the presence of unsaturated fatty acid. Holmgren (1902) concludes that the "T" substance is not fatty, and Bensley (1910) and Guilliermond (1933) come to the same conclusion.

Numerous amoebae were treated in accord with each of the methods named. In all, the outer layer of the refractive bodies give positive Golgi tests. In other experiments, described above, it was demonstrated that this layer contains fatty acid and protein. The outer layer of the refractive bodies therefore has the characteristics usually ascribed to Golgi bodies. Moreover, there are no other structures in the cytoplasm which have these characteristics. It is therefore highly probable that if *Amoeba proteus* possesses Golgi

substance it is in the refractive bodies. These bodies however contain structures which are not ordinarily found in Golgi bodies, namely, the shell and the central fluid. However, globules or vacuoles (which are sometimes themselves internally differentiated) are frequently found in Golgi bodies, e. g. the acroblast; and the structures in plant tissues most frequently considered to be the homolog of the Golgi bodies in animal tissues, viz. the plastids, quite commonly have an internal structure not unlike the shell and central fluid in the refractive bodies.

Zirkle (1926) says: "the ground substance of the chloroplast is in the form of a hollow, flattened, prolate spheroid surrounding a large central vacuole," and he holds that one or more vacuoles are present in the ground substance and that starch is formed in these vacuoles. Sharp (1934, p. 67) says: "A prevalent view regarding the interior of the chloroplast is that it consists of a somewhat denser cytoplasm, the stroma, in which there is a lipide carrying chlorophyll": and he says that different types of plastids are "characterized by different principal products of metabolic activity. In the higher plants and green algae this product is usually starch".

The facts that the central portion of the refractive bodies contain a carbohydrate and that the outer layer consists of a stroma which is impregnated with fatty substance, therefore indicate that they are in some respects related to plastids.

2. Vacuole Refractive Bodies.

The vacuole refractive bodies were studied in specimens treated in the same way as those in which the refractive bodies were studied. The following results were obtained:

In solutions containing neutral red the vacuole refractive bodies become crimson in color just as the refractive bodies do, but they stain much more rapidly and considerably more densely and all the substance in them stains, not merely a layer at the surface as in the refractive bodies. If pressure is applied to the cover-glass they spread and if it is released they round up like the refractive bodies, but there is no indication of any differentiation in the structure of different portions, i. e. they contain nothing in the nature of a shell. If pressure is applied and released several times in rapid succession vacuole refractive bodies which are in contact with each other fuse and form a large undifferentiated homogeneous mass which, when the pressure is released, assumes a globular form. The vacuole refractive bodies are therefore semi-fluid and elastic but homogeneous

in structure. They consequently differ radically in structure from the refractive bodies.

In nile blue, they become intensely blue; in neutral red, crimson: in brilliant cresyl blue, blue; and in osmic acid, black. In sudan dyes they do not stain unless they are first treated with phenol: and in fat solvents they disappear entirely. This shows that they contain fatty acid and other lipids, but that they do not contain neutral fat, protein or a carbohydrate which is insoluble in water. They are therefore like the outer layer of the refractive bodies in that they contain fatty acid and other lipids which stain crimson with neutral red, blue with brilliant cresyl blue, and black with osmic acid, and do not readily bleach with turpentine, hydrogen peroxide and chlorine water; but they differ from this layer in that they do not contain protein and are entirely soluble in fat solvents, e. g. trichloracetic acid.

In osmic acid the vacuole refractive bodies become black more rapidly than the refractive bodies and although they remain black much longer than the fat globules in the cytoplasm they bleach more rapidly in hydrogen peroxide and turpentine. This is what would be expected if the protein in the outer layer of the refractive bodies is in the form of a stroma and the lipids are imbedded in the interstices of this stroma, for this would retard the entrance of solutions which act on the lipids.

The facts that the vacuole refractive bodies become red in neutral red and black in osmic acid indicate they contain substance which is like that which Parat (1928), Hall (1929, 1930), Volkowsky (1933) and Finley (1934) call vacuome.

Blebs on the Crystals.

About five percent of the crystals have blebs attached. The blebs are globular in form with flat surfaces where they are in contact with other bodies. This shows that they are plastic. They vary greatly in size. The largest found were nearly as large as the largest crystals and the smallest were barely visible. They are usually on the under surface of the crystals, and they are consequently more readily seen if the main axis of the microscope is horizontal. This indicates that their specific gravity is greater than that of the crystals.

The blebs become intensely blue in nile blue and crimson in neutral red. They stain red considerably more rapidly than the

refractive bodies but not so rapidly as the vacuole refractive bodies. They become black in osmic acid and do not readily bleach in turpentine, hydrogen peroxide or chlorine water. The small ones become definitely dark in thirty minutes; the large ones require much longer. The former are soluble in trichloracetic acid, the latter are not. These facts indicate that the blebs change in composition as they develop and that the small ones have properties somewhat similar to those of the vacuole refractive bodies, and the large ones properties somewhat similar to those of the outer layer of the refractive bodies; and they suggest that the blebs form from the vacuole refractive bodies and give rise to the outer layer of the refractive bodies.

Fat.

Various investigators have observed that well fed specimens of *Amoeba proteus* usually contain in the food vacuoles and scattered thruout the cytoplasm, numerous globules which become black in osmic acid and red in sudan dyes; and they have found that in the absence of food these globules decrease greatly in number; but they gave no description of the globules other than the sui generis implication of the terms fat and oil. No observations have been reported on the origin of the fat beyond the fact that it in some way comes from the food in the food vacuoles; and no chemical changes have been observed in the process of digestion or synthesis of fat (Greenwood, 1886; Dawson and Belkin, 1928 and 1929; Mast and Hahnert, 1935).

With a view to obtaining more information concerning the nature of the fat we made the following experiments:

Some amoebae were fixed respectively in osmic acid, formalin and mercuric chloride and treated with various different fat stains. Others were put into culture fluid containing vital dye chrysoidine, and still others were subjected to low temperatures.

The following was found: The fat globules in the cytoplasm stained orange with sudan III in alcohol, red with sudan III in alcohol-acetone mixtures, red with sudan IV in alcohol, maroon with sudan IV in alcohol-acetone mixture, pink with nile blue sulphate, red with pure nile blue sulphate oxazone, brown to black with osmic acid (brown in 15 minutes and black in several hours), and yellowish with chrysoidine. The fat globules in the food vacuoles like those in the cytoplasm, stained with the sudan dyes, osmic acid and chrysoidine, but they stained blue instead of pink with nile blue sulphate.

In specimens subjected to a temperature of 5° C for several hours the globules in the cytoplasm crystallized but those in the food vacuole did not (Fig. 4).

The facts that the fat globules in the cytoplasm stain pink with nile blue sulphate and crystallize at 5° C and that those in the food vacuoles stain blue with nile blue sulphate and do not crystallize at 5° C indicate that the former are composed largely of neutral fat and the latter largely of fatty acids.

In staining solutions containing less than thirty percent water the fat globules dissolved. In fixatives containing chromates as the only oxidizing agent they were poorly preserved against dissolution in the higher alcohols. The significance of these reactions is not known.

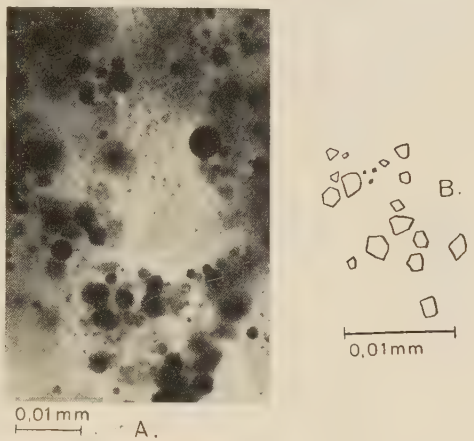

Fig. 4. A, photomicrograph showing the fat globules in a small region in a specimen of *Amoeba proteus* fixed in formalin stained with sudan IV and mounted in glycerine. The black circular areas represent fat globules. Note that they differ greatly in size and are very numerous. (Sudan IV, glycerine mount, 2 mm obj.-Zeiss Phoku camera). B, camera outlines of fat bodies after subjecting the organism to 5° C for twelve hours; mm, projected scale. Note the angular outlines indicative of crystallization.

Contractile Vacuole.

Metcalf (1910) demonstrated that the surface of the contractile vacuoles is well covered with a layer of granules and he concluded that the granules are involved in excretion. Nassonov (1924, 1925) using cytological methods studied the contractile vacuole in several protozoa and concluded that it is the homolog of the metazoan Golgi apparatus. There has been considerable controversy concerning the validity of these conclusions (Hall, 1929; Brown, 1930; Moore, 1934).

We made observations on *Amoeba proteus* with reference to these conclusions and found it to be an ideal organism for the investigation. The results obtained will be considered in a subsequent paper.

Organisms.

Numerous small rod shaped structures were found scattered fairly uniformly thru the cytoplasm in all specimens of *Amoeba proteus* examined. These structures look and stain like bacteria and are definitely distinguishable from the mitochondria. They are therefore probably bacteria. No attempt was made to culture them.

Mold spores of various kinds were frequently found in *Amoeba proteus*. These are usually engulfed like food but there is no evidence indicating digestion. They are usually retained for considerable periods of time and they often form in masses containing a hundred or more before they are voided. In a number of specimens spores were found with hyphae growing from them. Some of these hyphae branched and were in length equal to the diameter of the amoebae in which they were found. These hyphae were repeatedly seen to increase in length and in a few amoebae spores were found in all stages of development. Some spores obviously grow and fruit in the cytoplasm.

During several weeks a large percentage of specimens collected in a small portion of a permanent pond near Bar Harbor, Maine, had marked tufts of hyphae projecting from the posterior end. These hyphae were attached to groups of spores imbedded in the cytoplasm at the posterior end of the amoebae. The spores probably germinated in the amoebae and the hyphae formed probably broke thru the surface and then grew outside forming the tufts observed [1]).

The portion containing the spores to which these hyphae were attached was cut off in a number of specimens. This did not in any way interfere with locomotion and all the operated specimens lived for a number of days but they did not feed. However none of those with tufts attached had any food vacuoles or were observed to feed. It is therefore probable that they were in a pathological condition.

Leidy (1874) found in the neighborhood of Philadelphia a considerable number of amoebae which had at the posterior end long filamentous projections, forming a sort of caudal appendage. This appendage was essentially the same in all the amoebae which had it. In other respects some of the amoebae werelike *Amoeba proteus*: the rest were considerably smaller and differed in other respects. Owing to the presence of the caudal appendage Leidy held that

[1]) These observations were made jointly by Percy L. Johnson and S. O. Mast (Johnson, 1935).

the amoebae which have it are generically distinct and he consequently grouped them together under the generic name Ouramoebae, i. e. amoebae with tails (1879). Our observations demonstrate fairly conclusively that the caudal appendages observed by LEIDY were tufts of fungous hyphae and that there consequently is no foundation for the genus Ouramoeba.

No zoochlorellae were found either in *Amoeba proteus* or *Amoeba dubia*.

Summary.

1. The cytoplasm of *Amoeba proteus* contains numerous granules, some .25± μ in diameter (alpha granules, and others 1± μ in diameter (beta granules), plate-like and bipyramidal crystals, highly refractive, spherical bodies, some in the cytoplasm (refractive bodies) and others in the food vacuoles (vacuole refractive bodies).

2. Nothing is known concerning the structure and the composition of the alpha granules.

3. The beta granules vary in shape from nearly spherical to rod-like. They are elastic and they stain like the granules in other cells known as mitochondria. They tend to aggregate at the surface of the contractile vacuole.

4. There are two kinds of crystals, plate-like and bipyramidal. They are in vacuoles which contain fluid substance in which they are suspended. They vary in length from a maximum of 7 ± μ nearly to zero. The vacuoles vary in diameter from a maximum of 9 ± μ nearly to zero. The relation between the size of the vacuole and that of the crystal in it varies greatly. The substance in the vacuole is alkaline and it is under some conditions so fluid that the crystals are in violent Brownian movement and under others so viscous that no movement can be detected. Some have attached to them one or more blebs which are not optically active.

5. The plate-like crystals are all optically active but not on all axes. They are probably leucine.

6. The bipyramidal crystals are not optically active. Results obtained in spectroscopic analysis and observations made on their solubility and on their form show that they contain magnesium and have properties of glycine. They probably consist of a magnesium salt of a substituted glycine.

7. The refractive bodies are spherical in form. They vary in diameter from 15 μ nearly to zero. They consist of a hollow fragile shell filled with a fluid substance and a semi-fluid outer layer. The

outer layer stains crimson with neutral red. It is composed of a protein stroma impregnated with lipid containing fatty acid. It gives positive reactions to all important tests for Golgi substance. The shell is composed of a substance which has carbohydrate properties, but is not starch or glycogen. The composition of the central fluid is not known.

8. The vacuole refractive bodies vary in diameter from 10 μ nearly to zero. They stain crimson with neutral red like the outer layer of the refractive bodies. They are semi-fluid and elastic and homogeneous in structure. They are composed of lipids containing fatty acid. They do not contain neutral fats, proteins or carbohydrates.

9. About five percent of the crystals have blebs attached to their surface. These blebs change in composition as they develop. The smaller ones are somewhat similar to the vacuole refractive bodies and the larger ones somewhat similar to the refractive bodies. They probably form from the vacuole refractive bodies and give rise to the refractive bodies.

10. The fat globules in the cytoplasm stain pink with nile blue sulphate and crystallize at 5° C. The fat globules in the food vacuoles stain blue with nile blue sulphate and do not crystallize at 5° C. The former are composed largely of neutral fat and the latter largely of fatty acid.

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II.

Origin and Function Based on Experimental Evidence; Effect of Centrifuging on *Amoeba proteus.*

Outline.

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Introduction.

The views held concerning the origin and the function of various constituents of the cytoplasm of *Amoeba proteus* are based almost entirely upon the results obtained in observations on the location, the structure and the normal changes in structure of these constituents. Experimental evidence concerning the processes in question is therefore highly desirable.

Experimental methods have been successfully used in reference to functions of the nucleus (Verworn, 1909; Gruber, 1912; Stolc, 1910; Willis, 1916; Lynch, 1919). These investigators cut specimens in two in such a way that the two parts were essentially the same except that one contained a nucleus and the other did not, then they compared the two parts in reference to activity, response, behavior, respiration, feeding, digestion, reproduction and regeneration.

We applied this method to other structures and we also made direct observations on the development and the function of some of them in living specimens which were digesting various substances.

Methods.

It is well known that the specific gravities of the cytoplasmic constituents of cells differ considerably and that by means of the centrifuge they can be stratified without serious injury.

Harvey and Marsland (1929) found that this can be done in *Amoeba proteus* and we found that after stratification some of the constituents can be removed and others greatly reduced in number without serious injury, and that *Amoeba proteus* is consequently admirably adapted for experimental cytological investigation. The method of procedure follows:

Centrifuge tubes to fit the hematocrit head of an electric centrifuge were made from 3 mm. glass tubing. These were half filled with a 10 percent solution of pure soluble starch in culture fluid, then 25 to 50 cu.mm. of culture fluid containing 10 to 20 amoebae from a thriving culture were gently added to the solution in each tube. After the amoebae had settled to the surface of the starch solution a little of the two solutions at the interface was mixed by stirring with a glass needle so as to give a solution of graded density and viscosity [1]). The tubes were then attached to the centrifuge and rotated at various rates for different periods of

[1]) In previous work by other investigators the specimens were centrifuged either resting on the bottom of the tube or suspended in a sugar solution of graded

time, after which the amoebae were treated in one of three different ways as follows:

1. The amoebae were quickly taken from the tubes, mounted on slides and studied under low and high power, including immersion objectives.

2. The tubes were quickly removed, put into a freezing mixture and left until the temperature of the fluid in them decreased nearly to zero; then the amoebae were removed with a cold pipet and put into cold osmic acid, after which they were mounted on slides, studied and sketched.

3. The tubes were treated as in (2), then the amoebae were removed, put onto a cold glass Pfeiffer stage and cut as desired.

In addition to these methods the process of change in form and structure produced by centrifugal force was observed directly, by means of a Harvey-Loomis microscope centrifuge. The results obtained by the different methods will be presented under several different headings.

Effect of Centrifuging on *Amoeba proteus*.
Stratification of the Cytoplasm.

With the first method it was clearly seen that the amoebae became much elongated during rotation and that the cytoplasmic constituents aggregated in fairly definite zones; but it was found that the amoebae became active so soon after rotation ceased and that the stratification of the cytoplasm was so quickly lost after they had been mounted on slides that it was impossible to ascertain details concerning the distribution of the constituents.

With the second method it was found that if amoebae are very rapidly cooled immediately after rotation ceases and then kept cold they do not become active and stratification of the cytoplasm is retained; and that if, while they are in this condition, they are killed in osmic acid, permanent preparations can be made with no appreciable change in form or distribution of components.

By rotating specimens at different rates and for different periods of time, and then fixing them, a series of preparations was obtained comprising different stages in the process of change in form and structure produced by centrifugal force.

density. A sugar solution which is strong enough to suspend an amoeba is, however, owing to its high osmotic pressure, injurious to fresh water amoebae. The osmotic pressure of starch is so low that it has no appreciable effect on them.

This series of preparations shows that, as the duration or the rate of rotation increases, the amoebae elongate and as they elongate the fat globules aggregate at one end, the refractive bodies at the other end and the remaining cytoplasmic constituents in fairly definite layers between the two ends, and that elongation continues until the amoebae are separated into two parts, one containing the fat and other light [1]) substances, the other the refractive bodies and other heavy substances. Stratification of the cytoplasm is well illustrated in camera drawings reproduced in figure 1 A, B, C.

By referring to this figure it will be seen that beginning with the aggregation of fat globules at one end, the other cytoplasmic constituents, except the alpha granules, aggregate in layers in the following order: (1) crystal vacuoles without crystals, (2) crystal vacuoles with very small crystals and the contractile vacuole, (3) hyaline and very finely granular substance, (4) the nucleus and food vacuoles containing much fat and little starch, (5) crystal vacuoles with crystals of moderate size, (6) crystal vacuoles with large crystals, beta granules (mitochondria) and food vacuoles containing little if any fat, (7) refractive bodies. This shows that in reference to relative specific gravity these constituents fall into the order in which they are presented above, the first being the lightest.

The alpha granules did not aggregate in a layer. This is doubtless due to excessive friction owing to their minuteness. They were however much more abundant in the centrifugal half of the amoebae than in the centripetal half. It is therefore obvious that they are heavier than the fat, the substance in which the crystals are suspended, the contractile vacuole and the hyaline substances in the cytoplasm; but it is not certain what their specific gravity is in relation to that of the other constitutents.

Figure 1 D shows that in centrifuged amoebae the crystals, no matter what size, are all in close contact with the centrifugal surface of the crystal vacuole and that the chromatin granules in the nucleus are in the centrifugal portion of the nucleus. This

[1]) In observations on living amoebae in which the viscosity of the cytoplasm is low, it was found that the refractive bodies are invariably near the lower surface. This shows that these bodies are heavier than the other cytoplasmic constituents and that the end of the amoebae at which they aggregate during rotation on the centrifuge is consequently directed from the axis of rotation. This conclusion was confirmed by observations made on amoebae in the hematocrit tubes immediately after thay had been rotated and by observations on amoebae during the process of rotation in a microscope centrifuge.

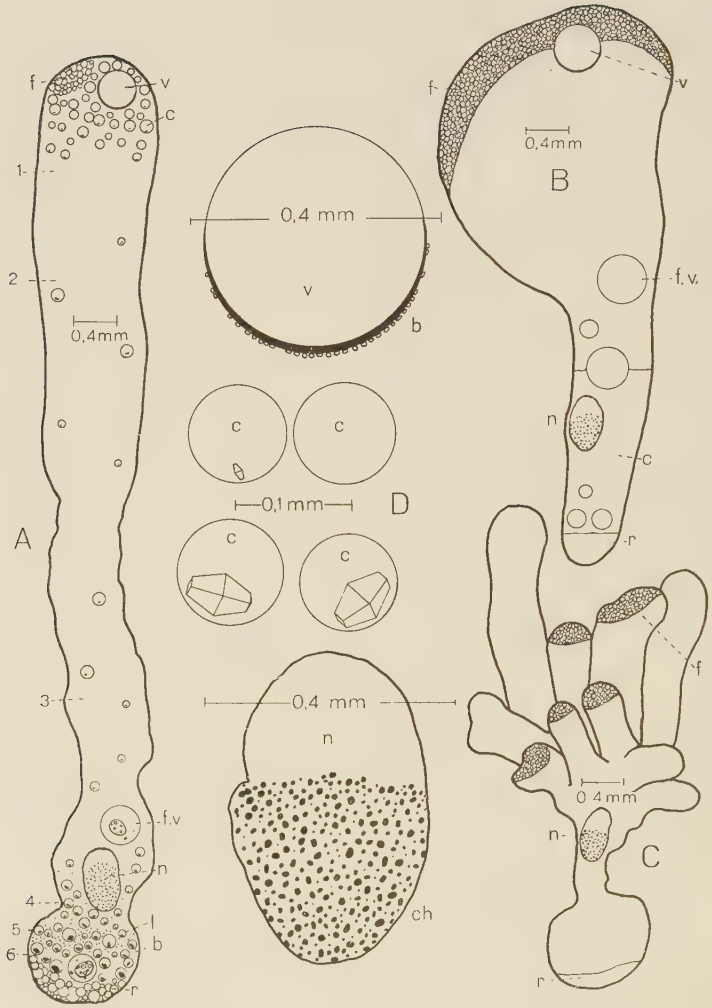

Fig. 1. Camera outlines of centrifuged specimens of Amoeba proteus and their contents. A, B, monopodal specimens; C, multipodal specimen; D, cytoplasmic constituents highly magnified; n, nucleus; ch, chromatin granules; v, contractile vacuole; b, beta granules; c, crystal vacuoles, some with crystals, some without; r, refractive bodies; f, fat globules; mm, projected millimeter scale; numerals 1—6, levels at which specimens used in operations were cut. — Note that during centrifuging the crystals shift toward the centrifugal surface of the crystal vacuoles, the chromatin granules toward the centrifugal surface of the nucleus and the beta granules and other substance to the centrifugal surface of the contractile vacuole. Note also that some of the crystal vacuoles have no crystals and that these and those with small crystals aggregate near the centripetal end, while those with large crystals aggregate near the opposite end.

shows that the crystals are heavier than the substance in which they are suspended in the crystal vacuoles and that the chromatin is heavier than the karyolymph.

Figure 1 D also shows that there is a layer of substance around the contractile vacuole and that this layer is much thicker on the centrifugal than on the centripetal side where it is very thin. The fact that this substance, during rotation, moves from the centripetal to the centrifugal surface shows that it is heavier than the surrounding substance and that it is relatively fluid, and it indicates that there is normally around the vacuole a layer of substance which differs considerably from that adjoining it. This layer is about 1 μ thick and it is usually fairly well covered with beta granules. In centrifuged specimens none of these granules are found on the centripetal side of the vacuole but they are fairly abundant on the opposite side. We were unable to ascertain whether or not this layer is the result of fusion of beta granules induced by the process of rotation.

In the observations made with the microscope centrifuge the changes in form and the movements of the different cytoplasmic constituents in the process of forming layers were very distinctly seen. Some of the specimens studied had no pseudopods and were much rounded, others had pseudopods in various numbers and sizes. The process of forming layers was essentially the same in both. Those which had no pseudopods gradually elongated and as they elongated they oriented with the longitudinal axes parallel to the long axis of the centrifuge cell. Those which had pseudopods usually turned until the tips of the pseudopods were directed toward the axis of rotation and then gradually elongated. In both, as they elongated, the refractive bodies were clearly seen to move toward the centrifugal end of the amoebae and the fat globules in the opposite direction. Not much in the way of directive movement was seen in the other constituents and the movement of the fat globules was much more striking than that of the refractive bodies. There were actual streams of fat globules, and in these streams aggregations were now and again formed in pockets, here and there, especially in small protuberances at the surface. However, the globules thus aggregated usually remained only a short time then broke away and rushed on again thru the cytoplasm. Thus they continued with spasmodic interruptions until they reached the end of the amoeba or the end of a pseudopod where they formed permanent aggregations (Fig. 1). After these aggregations had

formed, the tips of pseudopods containing them often separated from the rest of the body and then proceeded rapidly toward the axis of rotation. (This accounts for the fact that in a considerable number of specimens which were fixed after having been rotated no fat was found.) After the cytoplasm had become definitely stratified the amoebae continued gradually to elongate and as they elongated the central portion became smaller and smaller until it was reduced to a mere fiber which suddenly broke, after which the two ends rapidly separated [1]).

No detailed study of the energy required to stratify the cytoplasm was made, but in making the centrifuged preparations referred to above, the rate and the duration of rotation was recorded. Comparison of the extent of stratification of each of the preparations with the rate and the time it had been rotated shows that there is great individual variation but that the following statements hold for most of them. In these statements the force given was calculated from the rate and the radius of rotation.

With the force 27.5 times gravity and the time 2 minutes the amoebae are considerably elongated and there is definite indication of aggregation of the refractive bodies but no appreciable change in any of the other constituents. With the time 3 minutes the refractive bodies are closely packed at the centrifugal end and the crystals are clearly more numerous in the centrifugal half of the amoeba than in the other half. With the time 5.5 minutes the fat globules are clearly somewhat more abundant near the centripetal end than elsewhere.

With the force 350 times gravity and the time 5 minutes the fat globules are loosely aggregated at the centripetal end and the larger crystals at the opposite end next to the refractive bodies.

With the force 2700 times gravity and the time 3 minutes, most of the alpha granules are in the centrifugal half and the nucleus is near the center of the layer of large crystals. With the time 10 minutes about 35 percent of the amoebae are in two parts.

With the force 3575 times gravity and the time 8 to 10 minutes many of the crystals are out of the vacuoles and about 75 percent of the amoebae are in two parts.

[1]) These observations were made possible by the generosity of Prof. E. N. Harvey who put at our disposal his apparatus and assisted us in using it. We gratefully acknowledge our indebtedness to him.

Recovery of Stratified *Amoebae.*

If amoebae are centrifuged and mounted on slides at room temperature they recover so rapidly that no details can be seen concerning the process but if they are rapidly cooled immediately after they have been centrifuged in accord with method (3) and then mounted on a cold slide on a PFEIFFER stage in which the temperature can be controlled, the rate of recovery can be controlled as desired and in this way the process of recovery can be studied in detail under any magnification desired.

By means of these methods observations were made on the process of recovery in a considerable number of specimens in which stratification of the cytoplasm was in various stages. Without going into details concerning individual experiments the results obtained can be summarized as follows:

In specimens in which the cytoplasm is not very definitely stratified, the first indication of recovery consists of slight movement toward the centripetal end, of substance near the middle of the body. This movement then increases and extends very gradually and soon results in the formation of a pseudopod at the centripetal end and in definite forward streaming of cytoplasm along the entire longitudinal axis of the organism. At the posterior end of this stream the refractive bodies are aggregated. These bodies gradually enter the stream and are carried forward; and, owing to their high specific gravity, they become spread along the lower surface of the stream. After all of the refractive bodies have entered the stream, the crystals aggregated in the adjoining stratum enter it and are carried forward. As they are carried forward they tend to spread over the refractive bodies, owing to difference in specific gravity. Thus one stratum after another enters the stream and is carried forward, and as it is carried forward it gradually mixes with those which have gone before. This continues until the cytoplasmic constituents are uniformly distributed.

The fact that the process of locomotion is normal in stratified specimens shows that this process is not dependent upon distribution of the structures in the cytoplasm.

In specimens which have been centrifuged until the cytoplasm is very definitely stratified the process of recovery is essentially the same as it is in those in which the cytoplasm is not very definitely stratified, except that movement is in the opposite direction and the new pseudopods usually form a short distance from the

end, i. e., the new pseudopods form near the centrifugal in place of at the centripetal end.

If the beta granules and the hyaline substance have accumulated on the centrifugal surface of the contractile vacuole they rapidly become uniformly distributed over the surface; but if the nucleus has been definitely stratified it recovers very slowly if at all. In some specimens in which it was definitely stratified it did not recover in 24 hours, altho all these specimens recovered completely in other respects in a few minutes.

The time required for recovery depends upon the extent of stratification of the cytoplasm and the temperature. At room temperature, as previously stated, it requires only a few minutes even in specimens in which stratification is marked.

In all the specimens in which the process of recovery was observed, the plasmasol, when movement began, extended very nearly to the surface on all sides; that is, there was almost no plasmagel. This demonstrates that centrifuging breaks down the plasmagel and reduces its viscosity and that it probably also reduces the viscosity of the plasmasol [1]). This should be emphasized for it is of great importance in reference to one of the most prominent methods of measuring the viscosity of cytoplasm, namely, the so-called centrifuge method.

Origin and function of cytoplasmic constituents.

Methods.

Amoebae were centrifuged, cooled and mounted on a cold Pfeiffer stage in accord with method (3), then they were cut in various ways with a fine glass needle, either at once or after they had partially recovered. Some were cut immediately in front of the stratum containing the refractive bodies. In some of these all the refractive bodies and a very small amount of other substances were removed. Some were cut immediately back of the stratum containing the fat. In some of these all the fat except that in the food vacuoles was removed and only traces of other substances. Some were cut immediately in front of the stratum containing the larger crystals and most of the beta granules. In these, all the refractive bodies and nearly all the crystals were removed and the beta granules

[1]) Heilbrunn and Daugherty (1932) maintain that the plasmagel shifts toward the centrifugal end. Our observations indicate that if this obtains, the plasmagel disintegrates after it has shifted.

were greatly reduced in number. Some were kept, until in the process of recovery the stratum containing the refractive bodies had been carried forward in the plasmasol stream, and then cut in front of the stratum containing the larger crystals. In these most of the crystals and only a few if any refractive bodies were removed and the beta granules were much reduced in number.

Immediately after the operation each specimen was thoroly examined in reference to content of the cytoplasm, put into Chalkley culture solution in a dish and labelled. Chilomonads were then added to the solution in some of the dishes and bacteria to that in others, after which all of the specimens were examined from time to time for several days in reference to the behavior of the amoebae and the movements and changes in size and number of their cytoplasmic constituents. Approximately 700 specimens were thus cut and isolated, but in many of these the operation was for various reasons unsuccessful. Only those which lived more than 24 hours were studied.

The views of other investigators concerning the origin and the function of cytoplasmic constituents, details concerning the methods described above and other methods used by us and the results obtained thereby will be presented in the following pages under appropriate headings.

Refractive Bodies in the Food Vacuoles (Vacuome? Golgi Substance?).

The refractive bodies in the food vacuoles were designated "vacuome" by Volkonsky (1933) and "vacuole refractive bodies" by Mast and Doyle (1935). Mast (1926) maintains that they are stages in the process of digestion of food and that they function as reserve food. Mast and Hahnert (1935) demonstrated that they originate by coalescence of the so-called "neutral red granules" found in organisms ingested as food, and they maintain that during digestion they remain in the food vacuoles until all the other substance has disappeared, and are then discharged into the cytoplasm, owing to disintegration of the wall around the food vacuole. Horning (1926, 1928) contends that they are mitochondria and that they carry enzymes to the food.

We confirmed the contention that the vacuole refractive bodies originate from the food in the food vacuoles but not the contention that they are discharged, owing to disintegration of the wall of

the vacuole, and we found no evidence indicating that they are mitochondria or that they carry enzymes to the food.

Our observations were made as follows: Twenty-eight specimens were, at different times, cut in such a way as to remove the fat, the food vacuoles and all the refractive bodies in the cytoplasm. They were then put into a solution which contained chilomonads and left until they had ingested 5 to 15 each, after which they were washed in sterile Chalkley solution and mounted without food,

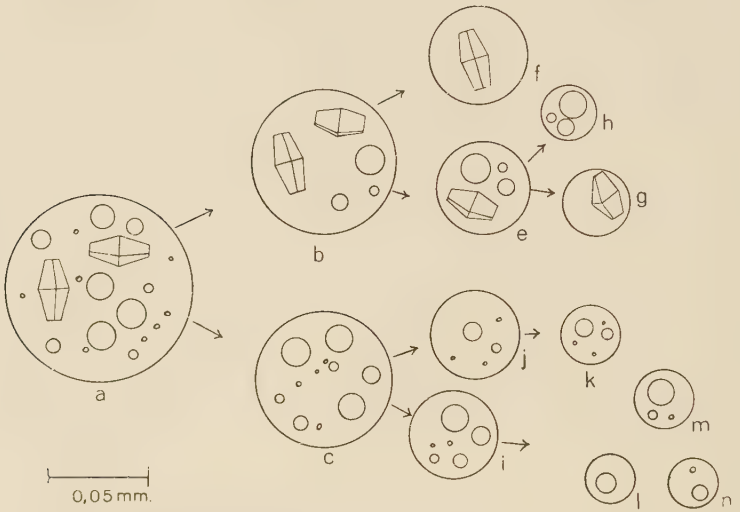

Fig. 2. Sketches showing division of a food vacuole containing crystals and vacuole refractive bodies, 16 to 30 hours after ingestion. Note that division results in the formation of some vacuoles which contain only crystals and others which contain only refractive bodies. Note also that the refractive bodies in the vacuoles decrease in size.

under cover-glasses supported with vaseline. Sixteen of these specimens were, under low and high magnification, examined hourly for 40 hours and twelve were observed continuously for 30 hours. The results obtained can be summarized as follows:

During the first 16 hours the changes observed in the food vacuoles were in full accord with the description of the process of digestion of *Chilomonas* given by Mast and Hahnert (1935). At the end of this time each food vacuole in all the specimens had 1 to 25 vacuole refractive bodies and some of them had crystals and irregular masses of substance which appeared to be indigestible. Shortly after this the food vacuoles began to divide. This continued

until each had divided several times. During the process of division of the vacuole, the refractive bodies, the crystals and the irregular masses of substance became separated, so that each vacuole finally contained only one of these three substances. The refractive bodies now gradually decreased in size in the vacuoles until they disappeared (Fig. 2). Not a single one of these bodies was seen to escape from the food vacuole by disintegration of the wall in accord with the contention of MAST and HAHNERT. Moreover, after all the food vacuoles had disappeared not a single one of them was found in the cytoplasm of any of the specimens.

MAST and HAHNERT did not carry their observations on digestion beyond 16 hours after ingestion. This doubtless accounts for their failure to observe the division of the food vacuoles and the decrease in the size of the vacuole refractive bodies in them.

The facts presented seem to prove that the vacuole refractive bodies disintegrate in the food vacuoles and that they get out into the cytoplasm by diffusion, i. e. that they originate, exist and disappear in the food vacuoles. Evidence will be presented later which indicates that after the substance of which they are composed has passed out of the vacuoles into the cytoplasm it is used in the development of the refractive bodies in the cytoplasm.

Refractive Bodies in the Cytoplasm (Golgi Substance?).

MAST and DOYLE (1935) demonstrated that the refractive bodies in the cytoplasm consist of a central fluid mass surrounded by a fragile shell covered with a layer of oily substance and that those in the food vacuoles are homogeneous in structure. They designated the former "refractive bodies" and the latter "vacuole refractive bodies".

CARTER (1863), WALLICH (1863), CALKINS (1905) and TAYLOR (1924) maintain that the refractive bodies are reproductive elements; GREEF (1866), STOLC (1902), VONWILLER (1918) and MAST (1926) that they are reserve food; BROWN (1930) that they are GOLGI substance; and MATTES (1924) that they are parasites; MAST and HAHNERT (1935) contend that they originate directly from the vacuole refractive bodies. There is consequently great diversity of opinion concerning their origin and function.

We made observations concerning these problems as follows:

Fifty three specimens which had been centrifuged were cut so as to remove the fat and all or nearly all of the refractive bodies.

They were then divided into three groups. The specimens in one group were washed and mounted in sterile culture solution under cover-glasses supported with vaseline. Those in another group were mounted in culture solution, containing numerous bacteria. Those in the remaining group were put into a solution containing chilomonads and left until each had ingested 6 to 13, then washed and mounted in sterile culture solution. All were then thoroly studied under high magnification at intervals of 12 hours. The results obtained in all the specimens in each group were essentially the same. Those obtained in a typical specimen from each are presented in table 1.

Table 1.

Origin and function of refractive bodies in *Amoeba proteus*.

Cut amoebae with few or no food vacuoles						
Time after operation, in hours	Fed on Chilomonas		Fed on bacteria		Not fed	
	Refractive bodies		Refractive bodies		Refractive bodies	
	Number	Diameter of largest in μ	Number	Diameter of largest in μ	Number	Diameter of largest in μ
0	0	—	0	—	—	—
12	0	—	0	—	—	—
24	?	—	20—25	5	10	6
48	30—35	2	?	—	?	—
72	30—35	8	20—25	6	6	6
96	—	—	20—25	7.3	3	6
120	—	—	—	—	2	7

This table shows that removal of all the refractive bodies does not seriously interfere with the vital processes in *Amoeba*, that in amoebae which contain no refractive bodies but contain food (chilomonads or bacteria) small refractive bodies appear in the cytoplasm in 24 to 48 hours and that these increase in size. It also shows that in amoebae with refractive bodies but without food the number of refractive bodies decreases.

Some detailed observations were made on the structure of refractive bodies in several starving amoebae. The results obtained in all are essentially the same. Those obtained in one follow: This specimen had only two refractive bodies. They were nearly the same in size and in structure. For 36 hours they had a well differentiated shell and a relatively thick outer layer (Fig. 3 A). Then for about 24 hours the shell gradually became less and less distinct until it disappeared, but the inner substance and the outer

layer were still differentiated (Fig. 3 B). Refraction had however descreased considerably. During the next 36 hours the inner substance increased in volume and the outer layer descreased in thickness and the surface of demarcation between them became irregular. This continued until the outer layer had disappeared (Fig. 3 E) and the refractive body became a vacuole which could not be distinguished from other fluid vacuoles in the cytoplasm.

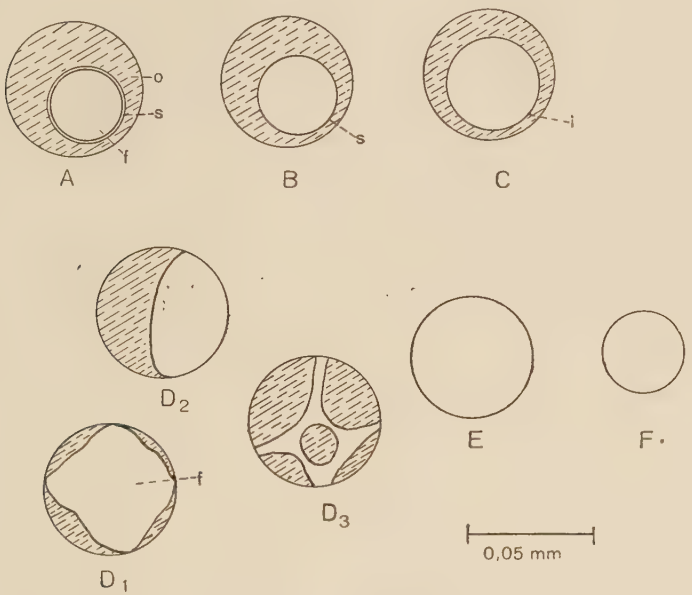

Fig. 3. Sketches showing stages in the disintegration of a refractive body. A—F, successive stages; A—D, optical sections; D_2, D_3, surface views; o, outer layer (cross hatched); s, shell; f, fluid; i, interface.

The results presented prove that the refractive bodies originate and develop in the cytoplasm, that this occurs only in the presence of food and that if food is absent those which are present desintegrate. The results consequently indicate that the refractive bodies in some way originate from the food and that they function as reserve food. They therefore support the contention of Greeff, Vonwiller and Mast that they are reserve food but they do not support the contention of Carter, Wallich, Calkins and Taylor that they are reproductive elements or the contention of Mattes that they are parasites.

The facts that the substance in the vacuole refractive bodies and that in the outer layer of the refractive bodies stain the same

with neutral red and other dyes and have the same physical properties, indicate that the latter is formed from the former.

We shall presently present evidence which indicates that substance in the bipyramidal crystals is also used in the development of the refractive bodies and that this substance and that obtained from the vacuole refractive body is transported by the beta granules.

Crystals.

Schewiakoff (1893) contends that the crystals in *Amoeba* are excretory products and Stolc (1902) that they are reserve food. Other investigators hold that they originate and function like the crystals in plants or those in other protozoa. There is however practically no evidence presented in support of any of these views.

We cut centrifuged amoebae so as to remove all the refractive bodies and a large proportion of the crystals and then treated and studied them as described above.

Many food vacuoles were found containing crystals varying in size from those barely visible to the largest seen. Some of these vacuoles were very closely observed at intervals for several days. Small crystals in the food vacuoles were repeatedly observed to become larger but not a single large one was observed to become smaller. The crystals found in old vacuoles were usually large. Vacuoles of this kind were repeatedly seen to divide in two in such a way as to separate the crystal from the rest of the contents as described above (Fig. 2). Some of the vacuoles thus formed contained nothing but crystals suspended in fluid. A considerable number of these vacuoles were closely observed periodically for several days and it was invariably found that the crystals decreased in size and finally disappeared leaving nothing but a fluid vacuole. This was observed to obtain both for pyramidal and for plate-like crystals. However, measurements were made only on the crystals in one amoeba. This amoeba contained three extraordinarily large pyramidal crystals which, owing to their size, could readily be distinguished from the other crystals present. One of these crystals was in a food vacuole. The other two were in crystal vacuoles. The results obtained are presented in table 2.

This table shows that the crystal in the food vacuole was, when found, 5 μ long and 3 μ wide, that it increased during the first day of observation to 7 μ long and 4 μ wide, that the food vacuole divided during the second day forming a vacuole which contained only the crystal and some fluid, and that from that time on the

Table 2

Change in the size of bipyramidal crystals in *Amoeba proteus*. f, in food vacuole; c, in crystal vacuole.

Time in days after first measurement	Size of three different crystals					
	A		B		C	
	Length in μ	Width in μ	Length in μ	Width in μ	Length in μ	Width in μ
0	7 c	5 c	5 f	3 f	4 c	3 c
1	5 c	4 c	7 f	4 f	2 c	1.5 c
2	3.5 c	2 c	7 c	4 c	—	—
3	2 c	1.5 c	4 c	3 c	—	—
4	—	—	2 c	1.5 c	—	—

crystal decreased in size. On the fifth day (when the observations were discontinued) it was only 2 μ long and 1.5 μ wide. The table shows that one of the crystals in a crystal vacuole decreased from 7 μ long and 5 μ wide on the first day, to 2 μ long and 1.5 μ wide on the fourth day and that it disappeared on the fifth day, and that the other crystal in a vacuole also decreased in size and disappeared.

These results strongly indicate that the crystals in *Amoeba proteus* are formed from food in the food vacuoles, that the crystal vacuoles are formed by division of old food vacuoles and that the crystals become smaller in the vacuoles and disappear; but they throw no light on the nature of the substance in the food from which the crystals are formed. The following experiment concerns this problem.

Amoebae which had been centrifuged and cut so as to remove most of the crystals were put into hypertonic solutions containing respectively different amino acids plus egg albumin. The amoebae were left in these solutions until they had ingested some of the fluid and formed vacuoles (Mast and Doyle, 1934), then they were mounted under cover-glasses and thoroly studied as described above. Bipyramidal crystals formed in some of the food vacuoles in the amoebae in all the solutions and optically active platelets, as well as bipyramidal crystals, formed in some of the food vacuoles of those in the solution which contained leucine. These results indicate that the crystals are normally formed from amino-acids derived from the food during the process of digestion.

We have no conclusive evidence concerning the function of the crystals but it was repeatedly observed that they decrease in number just before the refractive bodies increase in number. For example,

a cut specimen which immediately after the operation had 60 ± crystals and 13 refractive bodies, had, two days later, only 4 crystals and nearly 100 refractive bodies. These results seem to indicate that the substance in the crystals is used in the formation of refractive bodies.

Blebs.

Greeff (1866) and Vonwiller (1918) found that some of the crystals in *Amoeba* have blebs attached to their edges but they made no suggestions concerning their origin or their function.

We made numerous observations on the blebs on the crystals in normal amoebae and in amoebae which had been cut, and fed on *Chilomonas* or starved as described above, but obtained no clues concerning the origin or the function of the blebs. We found them as frequently on large crystals as on small ones. This indicates that they do not originate from substance liberated by the disintegration of the crystals and that they are probably in some way associated with substance in the food organism. We therefore made observations on the blebs in amoebae which had been in culture fluid containing various different chemicals.

After considerable experimentation we found that after they had fed for some time in culture solution containing 5 percent calcium gluconate, the blebs on the crystals increased enormously in number and the refractive bodies increased greatly in size.

To obtain more precise information concerning the effect of calcium gluconate on the blebs, it was added to one of two like cultures of *Amoeba dubia* [1]). After 18 hours 5 specimens were taken from each culture and mounted under cover-glasses. Then all the crystals in a given area were examined for blebs in each specimen. This was continued until 100 crystals had been examined in each specimen.

In the specimens from the culture fluid which contained calcium gluconate 14 percent of the crystals had blebs and each of these crystals had an average of 3 blebs. In the culture which did not contain calcium gluconate only 3 percent had blebs and none of these had more than one. That is, the calcium gluconate had caused the blebs to increase 14 times in number.

[1]) *Amoeba dubia* was used because it has relatively many more plate like crystals than *Amoeba proteus* and the blebs are much more readily seen on these than on the bipyramidal crystals.

These facts seem to indicate that the blebs are the result of adsorption of substance from the culture solution, and that certain substances in the solution cause increase in adsorption of substance normally only slightly adsorbed on the crystals. This conclusion is supported by the fact that the blebs are invariably found on the sharpest edges and corners of the crystals; for this, in accord with mechanical principles, is precisely where they would form if their formation is due to adsorption. Moreover, many crystals (10 to 15 percent) have rounded corners and edges. This can not be due to disintegration of the crystals for it was not found in crystals which were decreasing in size. It is therefore probable that it is due to adsorption of small quantities of substances on the corners and edges.

The blebs stain red with neutral red like the vacuole refractive bodies and the outer layer of the refractive bodies. This indicates that they consist of substance which had been in the vacuole refractive bodies and had passed into the cystoplasm when these bodies disintegrated — or that they are vacuole refractive bodies which have become attached to the crystals. Evidence presented elsewhere indicates that the former rather than the latter obtains.

Fat.

Mast and Hahnert (1935) demonstrated that the fat globules in chilomonads in food vacuoles in *Amoeba proteus*, pass from the chilomonads into the fluid in the vacuole and later disappear. They assume that the globules in some way pass *in toto* thru the wall of the vacuoles into the cytoplasm.

We centrifuged over 100 amoebae and removed from each the end containing the fat. Some of these amoebae were immediately treated with sudan III and others with nile blue sulfate. The rest were put into culture fluid containing numerous chilomonads and left until each had ingested several; then they were transferred thru several changes of sterile culture solution. After 30 minutes a few of them were treated with sudan III and a few with nile blue. This was repeated every half hour for 24 hours. All the treated specimens were immediately mounted under cover-glasses and examined under high magnification. No neutral fat or fatty acid was found in the cytoplasm of any of the specimens treated immediately after they had been cut. In those which were fed after the operation and then treated the following was found:

1. Thirty minutes after feeding: Numerous fat globules containing fatty acid in the chilomonads in the food vacuoles; no fat or fatty acid in the cytoplasm.

2. Two hours after feeding: Fat globules still in the chilomonads but they no longer contain fatty acid; no fat or fatty acid in the cytoplasm.

3. Three hours after feeding: Fat globules in the fluid in the food valuoles, none in the chilomonads: no fat or fatty acid in the cytoplasm.

4. Six hours after feeding: No fat globules in the food vacuoles; no fat or fatty acid in the cytoplasm.

5. Six to 15 hours after feeding: No fat or fatty acid in the food vacuoles or the cytoplasm.

6. Seventeen hours after feeding: Some indication of many very small neutral fat globules in the cytoplasm of some specimens.

7. Twenty hours after feeding: Numerous small neutral fat globules in the cytoplasm.

These results show that the fat globules in the chilomonads ingested contain fatty acid, that this is neutralized in the chilomonads in the food vacuoles, that the globules then pass from the chilomonads into the fluid in the food vacuoles, that they then disappear and that after this for several hours no fat or fatty acid is visible in the vacuoles or in the cytoplasm, after which fat appears in the cytoplasm in the form of numerous very small globules which increase in size. They do not show what becomes of the fat globules in the food vacuoles and how those in the cytoplasm are formed but they indicate that the fat in the vacuoles is hydrolized and saponified and that it passes out into the cytoplasm in solution and is there resynthesized and deposited. They lend no support to the contention of Mast and Hahnert that the fat globules pass *in toto* from the food vacuoles into the cytoplasm.

When amoebae are starved the fat in the cytoplasm rapidly decreases. It obviously functions as reserve food and in the production of energy.

Alpha Granules.

No evidence has been presented and no opinions have been expressed in the literature concerning either the origin or the function of the alpha granules. In centrifuged amoebae it was apparent, under high magnification that there were very few of these granules in the centripetal half of the amoebae. Because of their small size and the presence of the other constituents in the centrifugal portion

of the amoebae it was not possible to ascertain the exact region in which they were localized. We consequently obtained by these methods no information concerning their origin or function. And owing to their small size, we obtained none by direct observations on their location and their movement.

Beta Granules (Mitochondria).

It has been observed by a number of investigators that usually some beta granules are found on the surface of the contractile vacuole. Metcalf (1910) calls these "excretion granules" and those in the cytoplasm "cytomicrosomes". He maintains that those on the surface of the contractile vacuole form a clump during contraction, that the new contractile vacuole arises in this clump, that as the vacuole grows they become arranged in a layer on its surface and that they function in excretion of metabolic waste material. He says (p. 302): "There is, therefore, a constant relation between the excretory vacuole and a group of granules which surround it". (p. 307): "All the appearances seem to indicate that the excretory granules come from the ordinary cytomicrosomes [beta granules free in the cytoplasm] and are but slightly modified functionally from the ordinary cytomicrosomes".

Most investigators have been loath to accept Metcalf's interpretation of functional significance in the location of the granules on the surface of the contractile vacuole. They hold that the granules fortuitously come into contact with the contractile vacuole and then stick there and that their aggregation on the surface of the vacuole has no functional significance.

Fauré-Fremiet (1910), Arndt (1924) and Mast and Doyle (1935) maintain that beta granules are mitochondria, but they advance no opinions as to their function, and no one has presented any evidence concerning their origin.

We used two methods in making observations concerning the origin and the function of the beta granules. In one we attempted to ascertain the effect of removing various numbers of beta granules, on the length of life and the frequency of pulsation of the contractile vacuole. In the other we attempted to ascertain the function of the beta granules by making observations on their position and their movement in relation to the food vacuoles during digestion of food in them and the development of various structures in the cytoplasm. We shall describe these methods and the results obtained under the headings: Experiments and Observations respectively.

a) Experiments.

I.

Eighty-six centrifuged amoebae were cut at both ends so as to remove about the same relative amount of cytoplasm from each but so as to remove nearly all the beta granules from half of them (Fig. 1 A, cut at 1 and 3) and only about 15 percent of them from the rest (Fig. 1 A, cut at 2 and 5). All were now mounted in sterile culture fluid under cover-glasses and placed side by side in a damp chamber.

After 12 hours all those in the first group and only 13 of those in the second group were dead. Twelve hours later 9 of the original 43 in the second group were still alive and in some of these the number of beta granules had clearly increased considerably. But whether the new ones originated de novo or by division of old ones was not ascertained.

These results seem to indicate that beta granules are necessary for continued existence of *Amoeba*.

II.

Five or six centrifuged amoebae were cut at both ends somewhat as described above, mounted in sterile culture fluid under cover-glasses and examined under an oil immersion objective. Then the slide containing a specimen which had the desired protoplasmic constituents removed, was placed on a constant temperature stage at 22° and the maximum diameter and the frequency of contraction of the contractile vacuole ascertained. The whole process was then repeated with other cut specimens and with normal specimens from the same culture.

The maximum size of the contractile vacuole in the different specimens was so nearly the same that the variation in the volume of fluid eliminated per contraction was only about 3 percent and consequently negligible in reference to our conclusions.

The results obtained on frequency of contraction are presented in table 3. In this table the portions of the amoebae used are designated 1—7.

In 1 the two ends were removed, leaving about one-fourth of the amoeba where most of the beta granules and the nucleus had aggregated (Fig. 1). These specimens therefore contained relatively very large numbers of beta granules and the nucleus, but no contractile vacuole, no refractive bodies, no fat and very few crystals.

Table 3.

Relation between the number of beta granules and the rate of elimination of fluid by the contractile vacuole.

1—7, amoebae in which the number of beta granules per unit volume of cytoplasm varied inversely with the magnitude of the figures designating them.

The variation in the number of beta granules was obtained by cutting off different portions of the amoebae. Each average interval was obtained from ten consecutive contractions. The maximum size of the vacuoles varied but little. The volume of fluid eliminated was therefore nearly proportional to the frequency of contraction.

Designation of cultures	Designation of amoebae studied						
	1	2	3	4	5	6	7
	Average interval between contractions in minutes						
A	—	4.00	—	4.42	9.38	—	—
	—	4.35	—	5.17	10.50	—	—
B	3.50	4.08	4.50	4.75	6.73	—	—
C	3.42	4.18	—	4.50	7.16	20.72	25.05
Total Average	3.46	4.15	4.50	4.71	8.44	20.72	25.05

In 2 the lighter half was removed. These specimens contained a nucleus, all the refractive bodies, nearly all the alpha granules, most of the crystals and many beta granules, tho relatively fewer than the preceding, but no fat and no contractile vacuole.

In 3 the heavier half was removed. These specimens contained a nucleus [1]), no refractive bodies, very few alpha granules, few crystals, all the fat, the contractile vacuole and many beta granules, but relatively fewer beta granules than the preceding.

In 4 nothing was removed but the specimens were centrifuged. They contained all the beta granules but there were relatively fewer than there were in the preceding specimens.

In 5 the heavier two thirds was removed. These specimens contained no refractive bodies, no alpha granules and very few crystals but all the fat, the contractile vacuole, the nucleus, and some beta granules associated with the contractile vacuole, altho relatively many fewer than the preceding.

In 6 both ends were removed, leaving about one third of the entire body some distance from the lighter end. These specimens

[1]) By means of a glass rod, the nucleus was displaced from its usual position before the specimen was cut so that the nucleus was in the lighter half.

contained a nucleus but no refractive bodies, no fat, no contractile vacuole, very few crystals and very few beta granules.

In 7 all but the heavier one fourth was removed. These specimens contained all the refractive bodies, the nucleus, and a great many crystals but no fat, no contractile vacuole and almost no beta granules.

Examination of the descriptions of the contents of the amoebae used shows at once that those designated (1) had the highest number of beta granules per unit volume of cytoplasm, that those designated (2) had fewer and so on in the order given. That is, that the number of beta granules per unit volume of cytoplasm in the different amoebae consistently varied inversely with the magnitude of the numbers by which the amoebae were designated in the experiment and that this did not obtain for any of the other cytoplasmic constituents. By referring to table 3 it will be seen that the frequency of contraction of the contractile vacuole, also consistently varied inversely with the magnitude of these numbers. It is consequently obvious that the frequency of contraction varied directly with the number of beta granules per unit volume of cytoplasm and that they did not consistently vary with the number of any of the other consistents present.

The results presented therefore strongly indicate that the beta granules function in accumulation of the fluid eliminated by the contractile vacuole, i. e. they support Metcalf's contention that the beta granules function in excretion. This conclusion is supported by the facts that the contractile vacuole and the beta granules in amoebae stain respectively like Golgi substance and mitochondria in secretory cells in metazoa and are associated in the same way as these two structures in these cells (Nassonov, 1924, 1926; Brown, 1930; Mast and Doyle, 1935).

b) Observations.

Amoebae were centrifuged and cut in such a way as greatly to reduce the number of refractive bodies and crystals. They were then put into culture fluid containing chilomonads. As soon as one had ingested a chilomonad it was removed and mounted in sterile culture fluid under a cover-glass. This was continued until several had been mounted. Then each was thoroly examined under an oil immersion objective, every 15 minutes until the food vacuole had disappeared. This was repeated with other groups of amoebae until 12 had been examined.

Other specimens containing a single food vacuole were then isolated in the same way, but in place of making observations on them every 15 minutes during the entire course of digestion, they were left without observation for various periods of time, i. e. until the chilomonad in the food vacuole was in various stages of digestion, and then observed continously for 5 to 6 hours. Thirty specimens were studied in this way. The observations made on the thirty chilomonads in different stages of digestion in these specimens is equivalent to continous observation on 5 or 6 chilomonads during the whole course of digestion.

In all the observations made, special attention was given to the location and the movements of the beta granules. The temperature varied but little from 25°.

The phenomena observed were essentially the same in the different specimens, but there was considerable variation in the time at which they occurred. They can be summarized as follows:

1. Between 1 and 1.5 hours after ingestion the beta granules, moving about in the cytoplasm, frequently come in contact with the surface of the food vacuoles, crystal vacuoles and refractive bodies, but they do not aggregate there (Fig. 4 A). The fluid in the vacuoles changes from acid to alkaline. The ellipsoids[1]) in the chilomonads in the vacuoles become optically inactive.

2. Between 2 and 8 hours after ingestion the beta granules which come in contact with the surface of the food vacuoles frequently remain and aggregate in groups of 12 to 40, but these groups are not constant. Some of these granules are continually leaving and others arriving (Fig. 4 B). No beta granules are found adhering to the crystal vacuoles or the refractive bodies. The fatty acid in the fat globules in the chilomonads in the food vacuoles becomes neutralized and the globules pass out into the fluid in the vacuole and begin to decrease in size. The starch in the chilomonads changes to erythrodextrine. The chilomonads become globular.

3. Between 8 and 20 hours after ingestion no beta granules adhere to the surface of the food vacuoles, the crystal vacuoles or the refractive bodies. The starch in the food vacuoles disappears. The "neutral red" granules in the chilomonads coalesce to form vacuole refractive bodies. Crystals form in some of the vacuoles. The ellipsoids disintegrate.

[1]) The ellipsoids are two optically active bodies situated between the base of the pharynx and the nucleus (Mast and Doyle, 1935).

4. Between 16 and 30 hours after ingestion there are again groups of beta granules on the surface of the food vacuoles and in these groups some granules are continuously leaving and others arriving (Fig. 4 C, D). Beta granules occasionally adhere to crystal vacuoles. The food vacuoles divide forming vacuoles which contain only crystals, vacuoles which contain only vacuole refractive bodies and vacuoles which contain only indigestible substance. The vacuoles containing indigestible substance are voided. The vacuole

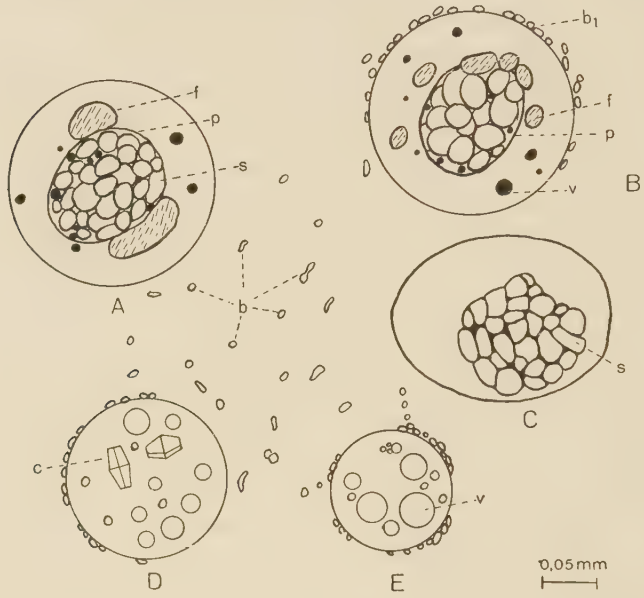

Fig. 4. Stages in the digestion of Chilomonas by Amoeba proteus. A, 2—6 hr.; B, 6—8 hr.; C, 8—16 hr.; D and E, 16—30 hr.; b, beta granules free in the cytoplasm; b_1, beta granules in contact with food vacuole; f, fat; s, starch; v, vacuole refractive bodies; c, crystals; p, pellicle at surface of Chilomonas.

refractive bodies and the crystals decrease in size and disappear leaving vacuoles filled with fluid. In some instances the crystals have blebs attached which increase as the crystals decrease. This was repeatedly observed but actual measurements were made on only two crystals. Each of these two crystals was at the beginning of the observations 2.5 μ long and each had a bleb attached which was 1 μ in diameter. Twenty two hours later both had decreased to 2 μ in length and the blebs attached had increased to 1.5 μ in diameter.

These results show that the beta granules move about in the cytoplasm and frequently come in contact with the other cytoplasmic constituents, that during the first stage of digestion of food in the vacuoles (2 to 8 hr.) they remain for some time in contact with the food vacuoles but not in contact with other constituents, that during the second stage (8—16 hr.) they leave immediately if they make contact, and that during the third stage (16 –30 hr.) they again remain for some time, in contact with the food vacuoles and now also with the crystal vacuoles and the refractive bodies. They show also that during the first stage, digestion begins and the fat leaves the vacuole; that during the second stage, the starch leaves the vacuole; and that during the third stage, the vacuole refractive bodies leave the vacuole, the crystals in the vacuoles increase in size and the refractive bodies in the cytoplasm increase in size. The beta granules therefore retain contact with the food vacuoles while the fat is leaving and while the vacuole refractive bodies are disintegrating, but not while the starch is leaving; and they retain contact with the crystal vacuoles while the crystals are decreasing in size; and with the surface of the refractive bodies, while the refractive bodies are increasing in size. These facts indicate that the beta granules function in transporting enzymes to the vacuoles and in transferring digested substances from the food vacuoles and the crystal vacuoles to the refractive bodies; and they support the conclusion presented above that the substance in the refractive bodies in the food vacuoles is used in the development of the refractive bodies in the cytoplasm.

The contention that the beta granules function in transporting enzymes and digested substances is supported by the evidence presented above, in favor of the conclusion that they serve as transporting agents in the process of secretion associated with the contractile vacuole.

If the number of beta granules in amobae is greatly reduced by operation a large majority of the amoebae die in the course of 24 hours, but in those which live the number of beta granules has clearly increased, so that there now appears to be as many as there were before the operation. Moreover, the frequency of contraction of the contractile vacuole has increased to normal, and since the frequency of contraction varies directly with the number of beta granules, this also indicates that the number of beta granules has increased. We have however no evidence which throws light on the origin of these granules or on the process of increase in number.

Summary.

1. In centrifuged specimens of *Amoeba proteus* the cytoplasmic constituents are fairly definitely arranged in strata as follows, beginning with the lightest: (1) fat; (2) crystal vacuoles (some with no crystals, others with minute crystals) and the contractile vacuole with a few beta granules adhering to it: (3) hyaline substance which becomes very finely granular after fixation, and a few scattered crystal vacuoles with small crystals; (4) the nucleus, some food vacuoles, crystal vacuoles with crystals of medium size, alpha granules and beta granules; (5) crystal vacuoles with large crystals, numerous beta granules, alpha granules and food vacuoles; (6) refractive bodies. The crystals in the vacuoles are at the centrifugal surface of the vacuoles. Their specific gravity is greater than that of the solution around them in the vacuoles. There are no chromatic granules at the centripetal surface of the nucleus. The specific gravity of these granules is greater than that of the karyolymph. There is a thick layer of substance with beta granules adhering to it at the centrifugal surface of the contractile vacuole and only an extremely thin layer at the opposite surface with no granules adhering to it.

2. Centrifuged amoebae recover very rapidly at room temperature but very slowly if at all at a temperature of about 5°. The nucleus recovers very slowly even at room temperature.

In recovery, pseudopods usually form at or near the lighter end of the amoebae; the refractive bodies then flow forward thru the center: then the largest crystals, etc. until all are thoroly mixed.

3. Centrifuged amoebae have practically no plasmagel. Centrifuging greatly reduces the viscosity of this layer.

4. All the fat and all the refractive bodies and a large proportion of the crystals can readily be removed by cutting off the two ends of centrifuged amoebae. This has no apparent injurious effect. Removal of a large proportion of the beta granules results in death of the organism.

5. The vacuole refractive bodies (Golgi substance?) form in the food vacuoles by coalescence of so-called "neutral red granules" in the food organism. They disintegrate in the vacuoles and the substance produced probably functions in the formation of refractive bodies in the cytoplasm.

6. The refractive bodies (Golgi substance?) originate and develop in the cytoplasm probably from substances obtained from

the vacuole refractive bodies and the crystals. They disintegrate during starvation. They function as reserve food.

7. The crystals originate and develop in the food vacuoles. They are probably formed from amino acids and magnesium salts obtained from the food. After they are fully formed the food vacuoles divide, the crystals go into some of the daughter vacuoles, the vacuole refractive bodies into others and the indigestible substance into still others. The crystals then decrease in size until they have disappeared, leaving a vacuole filled with fluid. The substance produced in their disintegration is probably used in the development of refractive bodies.

8. The blebs on the crystals stain red with neutral red like the vacuole refractive bodies and the outer layer of the refractive bodies. They probably originate by adsorption on the crystals of substance from the vacuole refractive bodies. Their function, if they have any, is not known.

9. Nothing is known concerning the origin or the function of the alpha granules.

10. The beta granules (mitochondria) are scattered thru all the cytoplasm but they are much more numerous at the surface of the contractile vacuole and at the surface of the food vacuoles at the initiation of digestion and toward the end of digestion when the vacuole refractive bodies disintegrate; and also at the surface of the crystal vacuoles when the crystals are decreasing in size and at the surface of the refractive bodies when they are increasing in size. There is a direct relation between the number of beta granules in the cytoplasm and the frequency of contraction of the contractile vacuole. They probably function in transferring substances from place to place in the cytoplasm.

Nothing is known concerning their origin.

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Vita.

William L. Doyle was born in Brooklyn, N. Y. on May 19, 1910. He was graduated from the primary and secondary schools of that city. In September 1927 he entered Dickinson College and studied there two years. In September 1929 he was admitted to The Johns Hopkins University as a New Plan Student in the Department of Zoology. The summer of 1930 was spent in investigation at the U. S. Bureau of Fisheries and as a student at the Marine Biological Laboratory. Woods Hole, Mass. This was made possible by a University Scholarship. A scholarship was again granted during the school year 1930—1931. The summers of 1931, 1932 and 1933 were spent as research assistant to Prof. S. O. Mast; the first at the Mt. Desert Island Biological Laboratory, the last two at the Marine Biological Laboratory. During the year 1931—1932 he was Student Assistant in Comparative Anatony and during 1932—1933 he held the same position in General Physiology. During the year 1933—1934 he was the recipient of a stipend from the Brooks Research Fund. The summer of 1934 was spent at the Carnegie Institution Biological Station at the Dry Tortugas. He was Bruce Fellow, 1934—1935.